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A real-time PCR method for the detection of *Salmonella enterica* from food using a target sequence identified by comparative genomic analysis

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ABSTRACT

A 5′-nuclease real-time PCR assay using a minor groove binding probe was developed for the detection of *Salmonella enterica* from artificially contaminated foods. *S. enterica*-specific sequences were identified by a comparative genomic approach. Several species-specific target sequences were evaluated for specificity. A real-time PCR assay was developed targeting a nucleotide sequence within the putative type III secretion ATP synthase gene (*ssaN*). An internal amplification control (IAC) probe was designed by randomly shuffling the target probe sequence and a single-stranded oligonucleotide was synthesized to serve as an IAC. The assay demonstrated 100% inclusivity for the 40 *Salmonella* strains tested and 100% exclusivity for 24 non-*Salmonella* strains. The detection limit of the real-time PCR assay was 41.2 fg/PCR with *Salmonella* Typhimurium genomic DNA and 18.6 fg/PCR using *Salmonella* Enteritidis genomic DNA; 8 and 4 genome equivalents, respectively. In the presence of a natural background flora derived from chicken meat enrichment cultures, the sample preparation and PCR method were capable of detecting as few as 130 *Salmonella* cfu/mL. Using the developed real-time PCR method to detect *Salmonella* in artificially contaminated chicken, liquid egg and peanut butter samples, as few as 1 cfu/10 g of sample was detectable after a brief (6 h) non-selective culture enrichment.

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1. Introduction

Salmonellosis, caused by infection with bacteria from the genus *Salmonella*, is one of the most common foodborne illnesses and is manifested by diarrhea, mild fever, nausea, and abdominal pains, with the symptoms developing in 12–72 h after consumption of contaminated food. In extreme cases it can also lead to death (Abubakar et al., 2007). *Salmonella* infection places significant health and economic burden worldwide (Anonymous, 2006; Kubota et al., 2008; Voetsch et al., 2004). In the United States alone, *Salmonella* infection causes an estimated 1.4 million foodborne illnesses annually (Voetsch et al., 2004), accounting for the largest number of outbreaks and outbreak-related cases (Lynch et al., 2006). In China, *Salmonellae* are the cause of approximately 40% of the incidence of bacteria-related food poisoning (Tong and Cheng, 2003). Food-associated Salmonellosis is most often associated with consumption of undercooked beef, poultry and eggs (DuPont, 2007). Within the last decade there has been an increase in the number of produce-associated *Salmonella* outbreaks (Sivapalasingam et al., 2004) and outbreaks associated with contaminated peanut butter (Anonymous, 2007, 2009).

The traditional methods used to detect *Salmonella* in food, which rely on laborious bacteriological and serological identification, can take 4–7 days to complete (Anonymous, 2008). Rapid and accurate detection of *Salmonella*, therefore, continues to be of considerable interest for both food safety surveillance and clinical diagnosis. Among the many rapid methodologies being developed for the detection of *Salmonella* and other foodborne pathogens, the polymerase chain reaction (PCR) has been frequently studied over the past decade because, in addition to being rapid and facile, the method can be highly specific and sensitive (Abubakar et al., 2007). Recently real-time PCR methods, in particular the 5′-nuclease assay, have gained popularity because the results can be monitored in real-time and the data can be analyzed quantitatively. Real-time PCR 5′-nuclease assay methods employ a fluorescently labeled oligonucleotide probe, in addition to primer-dependent amplification of target DNA, which can confer increased specificity. More recently, the linkage of a minor grove binding (MGB) moiety to the 3′-end of 5′-nuclease PCR probes has been employed to increase the stability of the probe/target interaction. This modification allows the use of shorter probes affording greater flexibility in probe design as well as the potential for increased PCR specificity and sensitivity.

Detection specificity, which depends on both the uniqueness of the sequence to the pathogen of interest, as well as the specific binding of

Abbreviations: BPW, buffer peptone water; GE, genome equivalent; IAC, internal amplification control; MGB, minor grove binding.

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the primers and probe to the target, is crucial to the efficacy of any PCR detection method. The target genes most frequently utilized for the specific detection of *Salmonella* are associated with virulence, including *invA* (*Salmonella* invasion protein gene) (Ferretti et al., 2001; Malorny et al., 2003; Rahn et al., 1992), *fimA* (major fimbrial subunit encoding gene) (Doran et al., 1994), *spv* (virulence gene) (Lampel et al., 1996), *stn* (enterotoxin gene) (Dinjus et al., 1997), *fliC* (flagellin gene) (Song et al., 1994), and *hilA* (invasion gene transcriptional activator) (Guo et al., 2000). Some of these genes have been reported to be absent in a few *Salmonella* serovars, whereas others yielded false positive results when detection methods based upon them were tested against non-*Salmonella* strains (Malorny et al., 2003; Moore and Feist, 2007; Rahn et al., 1992). Since most of the food inspection agencies have a zero tolerance policy for the presence of *Salmonella*, a lack of inclusivity and exclusivity of detection targets is one major obstacle toward the implementation and standardization of rapid molecular detection methods. Therefore, it is necessary to identify and carefully evaluate new targets for the detection of *Salmonella*. Most of the available target genes for PCR detection of foodborne pathogens, including *Salmonella*, were developed as the result of many years of work involving gene structure–function studies or immunological analysis. With the increasing availability of whole genome sequences, it is possible to couple bioinformatic tools for the identification of species-specific target genes with empirical verification in developing more specific and sensitive detection targets. Thus far, several studies have used comparative genomic tools to mine novel targets for pathogen detection using various algorithms (Kim et al., 2006, 2008; Liu et al., 2007; Ou et al., 2007), proving this to be a feasible methodology for the identification of unique and specific detection targets.

In this study we present a 5′-nuclease-MGB real-time PCR assay for the detection of *Salmonella* that utilizes primers and probes developed from a novel target sequence obtained by comparative genomic analysis. The efficiency, specificity and sensitivity of the PCR method were evaluated and the efficacy of the developed method was demonstrated for the detection of *Salmonella enterica* from artificially contaminated food samples.

Table 1
Bacterial strains used for specificity tests and real-time PCR.

Bacterial species (*n*)[a]	Strain(s)	PCR result
Salmonella enterica serovars		
Enteritidis (5)	ATCC BAA-1045; ATCC 13076; CDC H3502; CDC H3526; CDC H3527	+
Typhimurium (9)	ATCC 14028; CDC G7601; CDC G8430; CDC H2662; CDC H3278; CDC H3379; CDC H3380; CDC H3402; FSIS 26	+
Infantis (2)	ATCC 51741; F4319	+
Oranienberg	ATCC 9239	+
Anatum (2)	ATCC 9270; F4317	+
Montevideo (2)	ATCC 8387; FSIS 51	+
Saint Paul (2)	ATCC 9712; FSIS 39	+
Javiana	ATCC 10721	+
Chester	ATCC 11997	+
Bareilly	ATCC 9115	+
Muenchen	HFRV2C	+
Newport (2)	CDC H1073; H1275	+
Bredney	3V1PHE	+
Stanley	H0558	+
Kentucky (3)	FSIS 44(K); FSIS 62; FSIS 74	+
Heidelberg (3)	FSIS 109; FSIS 127; FSIS 134	+
Hadar	FSIS 44(H)	+
Thompson (2)	FSIS 120; FSIS 132	+
Aeromonas hydrophila	ATCC 7965	−
Brochothrix thermosphacta	ATCC 11509	−
Carnobacterium mobile	ATCC 49516	−
Campylobacter jejuni (2)	ATCC 33560; ATCC 35918	−
Citrobacter freundii	ATCC 8090	−
Enterobacter aerogenes	ATCC 129	−
Enterococcus faecalis	ATCC 7080	−
Escherichia coli O157:H7 (4)	ATCC 43888; ATCC 43889; ATCC 43890; ATCC 43895	−
Listeria innocua (2)	ATCC 33090; ATCC 57742	−
Listeria monocytogenes (4)	ATCC 19111; ATCC 19114; ATCC 19115; ATCC 19118	−
Staphylococcus aureus	ATCC 2593	−
Yersinia enterocolitica	GERP+	−
Yersinia intermedia	NRRL B-41442	
Yersinia kristensenii	NRRL B-41454	−
Yersinia pestis	Kuma (pCD1-minus)	−
Yersinia pseudotuberculosis	ATCC 29833	−

[a] *n* — number of strains tested.

2. Materials and methods

2.1. Bacterial strains

A total of 58 *S. enterica* strains and 22 non-*Salmonella* strains were used for pre-screening of *Salmonella* detection targets (Supplementary Data, Table S1). For real-time PCR specificity testing, 40 *Salmonella* strains and 24 non-*Salmonella* strains were tested (Table 1).

2.2. Preparation of genomic DNA

All bacterial strains were grown in Brain Heart Infusion (BHI) broth (BD, Bacto™, NJ, USA) overnight at optimal temperatures. DNA was extracted using the QIAGEN DNeasy® Blood and Tissue Kit (QIAGEN, MD, USA) either manually or using the QiaCube instrument according to manufacturer's instructions. DNA concentrations were determined spectrophotometrically using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, MA, USA).

2.3. Salmonella-specific target mining and primer and probe design

The workflow for selection of *S. enterica*-specific sequences is illustrated in Fig. 1, some of which was implemented in script written in the Perl programming language. Seventeen *S. enterica* genome sequences were obtained from NCBI (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/) and the Sanger Institute (www.sanger.ac.uk/Projects/Salmonella/) (Supplemental Table S1). The other 827 non-*Salmonella* bacterial

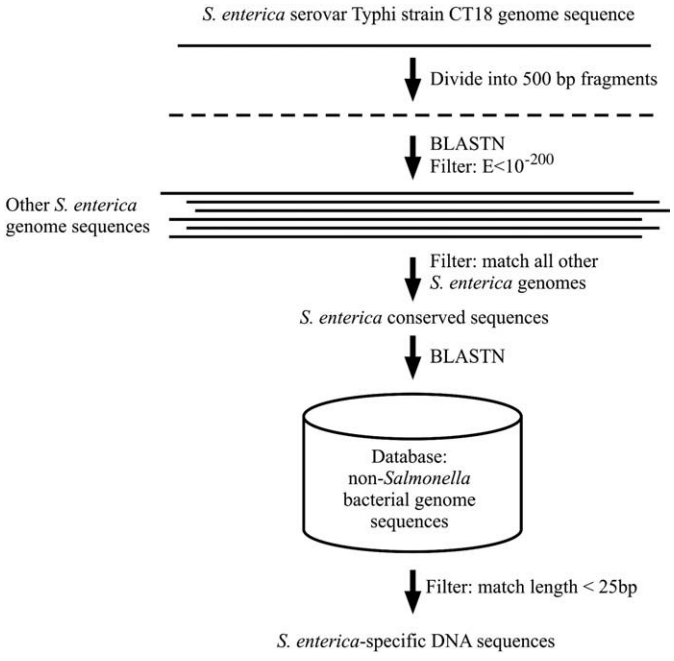

Fig. 1. Scheme for mining *S. enterica*-specific nucleotide sequences.

genomes used in this study were downloaded from the NCBI bacterial genome resource (ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria/). *S. enterica* serovar Typhi CT18 was selected as the reference strain and the genome sequence was cut into 500 bp fragments *in silico*. These fragments were then compared with the other *S. enterica* genomes by BLASTN (Altschul et al., 1997), and the fragments that matched all of the other *S. enterica* genomes with *E* values less than 10^{-200} were considered to contain *S. enterica*-conserved sequences. To determine the sequences specific to *S. enterica*, each conserved fragment was then searched against the database of non-*Salmonella* bacterial genome sequences using BLASTN. DNA fragments with a matched length less than 25 bp were identified as specific sequences for *S. enterica* and annotated by BLASTX against the nonredundant protein sequence database available at NCBI. Adjacent DNA segments were merged into longer fragments for primer design. As for the pre-screening of the potential detection targets, 22 fragments were randomly picked. Primers were designed using Primer Premier 5.0 (Premier Biosoft Intl., CA USA) or Primer Express® 3.0 (Applied Biosystems, CA, USA) software from these fragments and the specificity and sensitivity for each primer set was tested by end-point PCR and gel electrophoresis. One *Salmonella*-specific fragment was used to design a primer/probe set, namely c25 (Table 2), for the development of a real-time PCR assay. Primers were synthesized by Integrated DNA Technologies (IA, USA), and probes were synthesized by Applied Biosystems (CA, USA).

2.4. Real-time PCR conditions

Approximately 20 ng of genomic DNA was used in each 20 μL reaction containing 1×TaqMan® Gene Expression Master Mix (Applied Biosystems), 400 nM of each primer, 200 nM target probe and the optimal concentration of IAC probe and template (200 nM and 1200 copies/PCR respectively; described below). PCR was performed according to the following program: incubation for 2 min at 50 °C and then 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.5. Internal amplification control (IAC)

An internal amplification control probe was generated by randomly shuffling the sequence of the target probe. A 56-bp internal amplification control (IAC) oligonucleotide was then constructed by replacing the target probe sequence with the IAC probe sequence in the predicted amplicon (Table 2). The IAC oligonucleotide and probe were synthesized by Integrated DNA Technologies.

2.5.1. Verification of IAC detection

To verify that the IAC template and probe combination yielded a detectable product, real-time PCR assays were performed using a 7500 Real Time PCR System (Applied Biosystems) with 200 nM of each primer, 200 nM IAC probe, and 1.2×10^3 copies of IAC oligonucleotide. In a separate experiment 100 pg of *Salmonella* genomic DNA was tested in the absence of the IAC template to confirm that the IAC fluorescence was not due to amplification from *Salmonella* genomic DNA.

Table 2
Primer and probe sequences.

Primer/probe	Sequence (5′–3′)
c25	Forward: GCCAGAAGCGTGCTTTTCC
	Reverse: GGGCAACGAGTGGGTATTTTT
	Probe: FAM-CACGCCAGGAGCAG-MGB
IAC probe	VIC-GACGGAGACCAGCC-MGB
IAC[a]	GCCAGAAGCGTGCTTTTCCCGACGGAGACC AGCCAAAAAATACCCACTCGTTGCCC

[a] The sequence of the IAC probe is underlined.

2.5.2. IAC detection limit

The IAC detection limit was tested in triplicate using decreasing amounts (1.2×10^5, 1.2×10^4, 1.2×10^3, 1.2×10^2, 1.2×10^1, and 1.2×10^0 copies/reaction) of IAC oligonucleotide.

2.5.3. Target-IAC co-amplification

To confirm target and IAC DNA were capable of being co-amplified, reactions were set up with 400 nM of each primer, 200 nM target probe, 200 nM IAC probe, 100 pg *Salmonella* Enteritidis DNA, and optimal amount (the lowest detectable concentration×10) of IAC.

2.5.4. IAC probe optimization

In experiments designed to optimize the concentration of IAC probe, the reaction mixtures included 400 nM of each primer, 100 pg *Salmonella* genomic DNA, the optimal amount of IAC, and decreasing concentrations (400 nM, 200 nM and 100 nM) of the IAC probe.

2.5.5. Inhibition of target amplification by the IAC

To determine the highest IAC concentration that did not inhibit target amplification, PCR assays were performed with 400 nM of each primer, 2.5 pg *Salmonella* genomic DNA [~500 genome equivalents (GE)] (McClelland et al., 2001), IAC probe at the optimal concentration, 200 nM *Salmonella* target probe, and the IAC oligonucleotide at increasing concentrations (1.2×10^0, 1.2×10^1, 1.2×10^2, 1.2×10^3, 1.2×10^4, and 1.2×10^5 copies/reaction).

2.6. PCR specificity, sensitivity, and efficiency

Specificity of the primer/probe was tested against a total of 40 strains of *S. enterica* and 23 bacterial strains from 11 other genera (Table 1). For sensitivity testing, genomic DNA from *S.* Enteritidis (CDC H3526) or *Salmonella* Typhimurium (CDC G7601) was ten-fold serial diluted and tested by real-time PCR. Sensitivity evaluations were done twice in triplicate, with the presence of internal amplification controls. PCR efficiencies were calculated from the standard curves using the following equation:

$$PCR\,efficiency = [10^{(-1/M)}]-1$$

where M is the slope of the standard curve (Rasmussen, 2001).

2.7. Detection of S. enterica in the presence of background flora

To investigate the capability of the assay to detect *Salmonella* in the presence of the background flora, exponential phase cultures of *S.* Typhimurium, *S.* Enteritidis and *S.* Infantis were mixed, serial diluted to ~1–10, 10–100, 100–1000, 1000–10,000 cells/100 μL, added into 900 μL of an overnight enrichment culture generated by incubating 10 g of uninoculated raw chicken with 90 mL of buffered peptone water (BPW) at 37 °C with shaking (130 rpm). The chicken sample was confirmed to be *Salmonella* free by standard microbiological methods prior to use for experiments and PCR. DNA was extracted from the mixture, and used for real-time PCR. The number of *Salmonella* cells used for the inoculum was determined experimentally by first counting the mixed culture in a Petroff-Hausser Counting Chamber and confirmed by 6×6 drop plating (Chen et al., 2003) on BHI agar. A serial dilution *S.* Typhimurium (CDC G7601) genomic DNA was used to generate a standard curve, for comparison with which the number of *Salmonella* genomic equivalents in the samples was estimated using the 7500 SDS software.

2.8. Artificial contamination of food samples

2.8.1. Chicken

Equal amounts of *S.* Typhimurium, *S.* Enteritidis and *S.* Infantis overnight cultures were mixed together. The cocktail was ten-fold

serial diluted and enumerated in a Petroff-Hausser Counting Chamber and then confirmed by plating on BHI using the 6×6 drop plating method (Chen et al., 2003). Chicken breasts were purchased from a local grocery store, frozen and thawed at 4 °C overnight, confirmed to be *Salmonella* free by standard culture methods (USDA-FSIS) prior to use for experiments. Samples were cut aseptically into 10 g pieces, inoculated in triplicate with approximately 1, 10, 100 *Salmonella* cells per gram, placed into Stomacher bags and refrigerated at 4 °C overnight. After the overnight storage, 90 mL Difco™ buffered peptone water (BD, NJ, USA) was added to each bag and the samples were mixed using a Stomacher for 2 min at medium speed. The samples were then incubated for 6 h at 37 °C with gentle shaking (130 rpm). DNA was extracted from 1 mL of the enrichment with the Qiagen DNeasy Blood and Tissue Kit in a semi-automated QiaCube instrument and used as template for real-time PCR. PCR results for each sample were verified by standard culture methods.

2.8.2. Liquid egg

Pasteurized liquid egg was kindly provided by Michael Foods Inc. (MN, USA). Ten mL of liquid egg was transferred into a Stomacher bag, inoculated with 100 μL *S. enterica* culture and refrigerated overnight. As described above for the chicken samples, 90 mL BPW was added to the samples which were mixed using a Stomacher for 2 min at medium speed. The samples were incubated at 37 °C for 6 h. One mL from each enriched sample was used for DNA extraction and the extracted DNA was subsequently used for real-time PCR. Sample enrichments were incubated for an additional 18 h (24 h total) to be tested for *Salmonella* using standard culture methods (USDA-FSIS). Control samples that were not inoculated with *Salmonella* were plated on BHI agar before and after enrichment to enumerate the background flora. This experiment was performed in triplicate and repeated twice.

2.8.3. Peanut butter

Peanut butter was purchased from a local grocery store. Ten grams of peanut butter was artificially contaminated with *S. enterica*, samples were cultured using BPW, DNA was prepared, and real-time PCR was conducted following the methods described above, except that samples were used directly after inoculation and mixing.

3. Results

3.1. The mining and preliminary screening of Salmonella-specific detection targets

A set of 9619 sequences were obtained by dividing the genome sequence of *S. enterica* serovar Typhi CT18 into 500 bp fragments. A total of 6987 showed high sequence identity to the other 16 *S. enterica* genomes (i.e., *E* values less than 10^{-200}). To identify fragments specific to *S. enterica*, the 6987 conserved fragments were screened for nucleotide sequence similarity against the 827 non-*S. enterica* bacterial genomes available at NCBI. Filtered by the predetermined criteria, a total of 361 fragments with matched nucleotides less than 25 bp in length were further identified as specific to *S. enterica*. The genomic location of these *Salmonella*-specific sequences in the *S. enterica* serovar Typhi CT18 genome and their predicted gene products are provided as supplementary data (Table S2 in Supplementary Data).

Contiguous fragments were combined and 22 merged fragments were randomly picked to design 22 primer sets that were further used in the preliminary screening of *S. enterica*-specific targets by end-point PCR and gel electrophoresis (Table S3 in Supplementary Data). Twelve of the 22 primer sets exhibited 100% inclusivity for the 58 *Salmonella* genomes and 100% exclusivity of 22 non-*Salmonella* genomes (Table S3 in Supplementary Data). The other ten primer sets either failed to generate an amplicon from one or more *Salmonella* genomes, or produced amplicons of the predicted target size from genomic DNA from bacteria other than *Salmonella*. One *S. enterica*-specific sequence (fragments 105–106, Table S2) was used to design a primer/probe set (c25, Table 2) for real-time PCR.

As indicated in the supplementary material, the c25 primers target a nucleotide sequence within *ssaN* putative gene encoding a protein that is part of the type III secretion ATP synthase within the *Salmonella* pathogenicity island 2 (Hensel et al., 1997).

3.2. IAC optimization

The amount of internal amplification control was optimized so that an IAC signal could be detected without inhibiting amplification of the target sequence. First, real-time PCR detection of the IAC was studied in the absence of *Salmonella* genomic DNA using 200 nM IAC probe at varying concentrations of IAC template. When the IAC was present at 1.2×10^1 copies per assay, one out of three assays yielded a positive result ($C_T = 39.68$). With the presence of 1.2×10^2 or more copies of the IAC, all three parallel assays generated positive result ($C_T = 36.56 \pm 0.89$). IAC probe concentration was then optimized in a duplex reaction containing 1.2×10^3 copies of the IAC and 180 pg *Salmonella* genomic DNA. The maximal VIC signal (IAC) was generated at an IAC probe concentration of 200 nM without affecting the FAM signal (target). Therefore 200 nM was chosen as the concentration of the IAC probe. IAC inhibition of *Salmonella* target amplification was tested by adding varying amounts of IAC template in the duplex reaction. The target C_T value (30.65 ± 0.17) remained unaffected within a broad range of IAC concentrations (0 to 1.2×10^4 copies/reaction). However, the target amplification signal decreased when the IAC concentration was 1.2×10^5 copies/assay or higher. Thus, an IAC template of 1.2×10^3 copies/assay was used in all additional experiments.

3.3. Test specificity

Forty *S. enterica* strains and 24 bacterial strains from other genera were used to test the specificity of the c25 real-time PCR assay. The results are shown in Table 1. All genomic DNA templates from *S. enterica* strains yielded a detectable amplicon, whereas those from all non-*S. enterica* strains generated only the IAC signal; thus, the c25 primer/probe set was specific for the detection of *S. enterica*.

3.4. Limit of detection

S. Typhimurium (CDC G7601) genomic DNA was 10-fold serial diluted and used to test the sensitivity of the real-time PCR assay. Results demonstrated that PCR was capable of detecting as low as 41.2 fg of *Salmonella* genomic DNA per assay, which is approximately of 8 GE. The PCR efficiency was 95.3%. No IAC signal was detected when the target concentration exceeded 412 fg/reaction. Using *S.* Enteritidis (CDC H3526) genomic DNA as the template, a detection limit of 18.6 fg/assay was observed (approximately 4 GE), with a PCR efficiency of 97.1%. However, IAC amplification was inhibited when more than 18.6 pg target template was present in the reaction.

3.5. Detection of Salmonella in the presence of background flora

The sensitivity of the real-time PCR detection of *Salmonella* in the presence of background flora was examined by combining various dilutions of *Salmonella* with an overnight enrichment culture generated using an inoculated chicken sample. The total aerobic plate count from the overnight enrichment culture was 1.1×10^7 cfu/mL. Even in the presence of background flora, real-time PCR was capable of detecting as few as 130 cfu *Salmonella*/mL (Table 3), but at this concentration of cells only half of the assays yielded a positive result. This was probably due to the fact that at this cell concentration, there would be only approximately 4 *Salmonella* GE/assay; very near the

Table 3
Detection of *Salmonella enterica* in the presence of background flora.

Salmonella cells (cfu/mL)	Mean C_T	*Salmonella* genomes GE/mL[a]
0	>40	Not detected
1.30×10^2	38.86[b]	1.96×10^2
1.30×10^3	36.26	1.04×10^3
1.30×10^4	32.24	1.35×10^4
1.30×10^5	29.32	8.75×10^4
1.30×10^6	25.31	1.14×10^6

[a] The number of *Salmonella* genome equivalents (GE) per mL was determined by comparing the C_T values to the standard curve and then multiplying the GE by 50, because 2 µL of the 100 µL DNA prep was used in the PCR assay.
[b] 50% of the samples yielded a positive result.

limit of detection of the PCR assay. At all cell concentrations greater than 1.3×10^2 cfu/mL, the PCR method was able to detect the *Salmonella* in the presence of the background flora (Table 3). For all samples that yielded a positive result, the estimated number of genomic equivalents was close to the number of *Salmonella* cells that were actually added in the enrichment before DNA extraction (Table 3). Samples that were not inoculated with *Salmonella* did not yield a fluorescence signal by PCR (C_T>40).

3.6. Detection of Salmonella in artificially contaminated food

After 6 h of enrichment, real-time PCR generated positive results from artificially contaminated chicken meat, egg, and peanut butter, even at inoculum levels <10 cfu/sample (Tables 4–6). One chicken sample (~6 cfu /sample; Table 4) and one peanut butter sample (~9 cfu /sample; Table 6) did not yield positive results by the PCR method. For the single chicken sample that gave a negative result, a failure was observed in the semi-automated DNA extraction. The prepared sample contained no measurable DNA which was most likely the cause of the false negative result. For the peanut butter sample that yielded a negative result, no amplification of the IAC was detected, suggesting that PCR amplification may have been inhibited. All samples yielded positive results when tested using the microbiological methods as described in the USDA-FSIS Microbiological Laboratory Guide (MLG 4.04). Thus, the consistency between PCR detection and the standard microbiological methods was 97.9% (94/96).

As expected, increased sample inoculums resulted in a lower C_T in the real-time PCR detection assay. Since the high concentrations of background organisms in enriched food samples prevented the direct determination of the concentration of *Salmonella* by microbiological methods, the number of *Salmonella* GE/mL was estimated from the real-time PCR results and are presented in Tables 4–6. The estimated GE after 6 h enrichments were consistent with increases in the initial inoculum in artificially contaminated chicken and liquid egg samples, whereas more variability was observed with the peanut butter samples.

Table 4
Detection of *Salmonella enterica* from artificially contaminated chicken meat.

Inoculum (cfu/sample)	Detection results[a]		$C_T\pm$SD ($n=3$)	GE/mL after enrichment
	Microbiological (USDA MLG 4.04)	PCR		
0	0/6	0/6	–	–
5	3/3	3/3	33.03 ± 1.92	5.67×10^4
6	3/3	2/3[b]	32.81 ± 0.91	6.55×10^4
50	3/3	3/3	29.82 ± 0.80	9.1×10^5
64	3/3	3/3	29.49 ± 0.14	4.38×10^5
500	3/3	3/3	26.10 ± 0.92	3.26×10^6
645	3/3	3/3	27.92 ± 0.60	1.16×10^6

[a] Positive results/samples tested.
[b] A failure was observed in the DNA extraction.

Table 5
Detection of *Salmonella enterica* from artificially contaminated liquid egg.

Inoculum (cfu/sample)	Detection results[a]		$C_T\pm$SD ($n=3$)	GE/mL after enrichment
	Microbiological (USDA MLG 4.04)	PCR		
0	0/9	0/9	–	–
1	3/3	3/3	35.62 ± 0.11	1.01×10^4
10	3/3	3/3	34.47 ± 1.19	2.39×10^4
15	3/3	3/3	32.40 ± 1.24	6.07×10^4
19	3/3	3/3	34.22 ± 0.83	1.69×10^4
100	3/3	3/3	32.20 ± 0.22	8.09×10^4
150	3/3	3/3	30.10 ± 0.39	2.11×10^5
189	3/3	3/3	31.63 ± 0.07	7.93×10^4
1500	3/3	3/3	28.66 ± 1.29	6.13×10^5
1892	3/3	3/3	29.40 ± 0.23	3.24×10^5

[a] Positive results/samples tested.

4. Discussion

Whereas most of the target genes used for PCR detection of foodborne pathogens encode proteins involved in virulence and were identified as the result of many years of work involving gene/protein structure–function studies, the progress in computational genomics has led the way to more efficient and customized mining of genomes for species-specific nucleotide sequences. Several software packages for such data mining have been developed based upon sequence alignment (Lu et al., 2006; Yao et al., 2007). Here we have employed a novel comparative genomics approach to identify nucleotide sequences for the specific detection of *S. enterica*. In addition to identifying unique genes that are required for virulence or other specialized metabolic functions, this genome mining approach allows for the identification of unique genes and sequences for which a function has not yet been assigned. Furthermore, in our method, the reference genome was divided into shorter (500 bp) segments rather than complete gene sequences, which also increases the likelihood of revealing specific sequences within intergenic regions or spanning two genes. The importance of identifying such sequences as potential species-specific targets is exemplified by the fact that the *Listeria monocytogenes* target sequence used in the popular BAX® PCR System (DuPont-Qualicon, DE, USA) encompasses part of the putative gene *lmo2234* and additional non-coding sequence upstream of this gene (Zhang et al., 2004). Interestingly, among the 361 *S. enterica*-specific sequences identified in this study, 49 were designated hypothetical proteins and 130 were identified as putative genes for which little or nothing is known about the function of their gene products. In addition to their use as potential species-specific nucleotide sequences for PCR identification of *S. enterica*, the identification of these species-specific hypothetical protein encoding regions might also be helpful in

Table 6
Detection of *Salmonella enterica* from artificially contaminated peanut butter.

Inoculum (cfu/sample)	Positive detection results[a]		$C_T\pm$SD ($n=3$)	GE/mL after enrichment
	Microbiological (USDA MLG 4.04)	PCR		
0	0/9	0/9	–	–
3	3/3	3/3	27.74 ± 1.09	1.15×10^6
4	3/3	3/3	35.29 ± 0.75	1.31×10^4
9	3/3	2/3[b]	34.11 ± 0.76	1.77×10^4
27	3/3	3/3	27.15 ± 0.16	1.42×10^6
40	3/3	3/3	32.94 ± 0.19	5.15×10^4
88	3/3	3/3	30.21 ± 0.46	1.98×10^5
273	3/3	3/3	23.88 ± 0.02	1.01×10^7
398	3/3	3/3	29.93 ± 0.39	3.23×10^5
875	3/3	3/3	29.23 ± 0.91	3.99×10^5

[a] Positive results/samples tested.
[b] A failure in IAC amplification was observed.

future gene structure–function analysis and ultimately benefit the understanding of the metabolic behaviour unique to *S. enterica*.

The specificity of the target sequences identified *in silico* is largely determined by the screening criteria (e.g., *E* values) and the availability of reference genomes. Undoubtedly the expansion of genome databases will inevitably result in the elimination of some nucleotide targets previously considered specific. Thus, empirical verification with a large collection of bacterial strains is absolutely critical. In this study, the specificity of 22 of the 361 *S. enterica* target sequences was tested by end-point PCR using both standard cultures and isolates of various origins in China. The fact that 12 of the 22 target sequences were found to be specific demonstrated both the effectiveness of the comparative genomics approach for identifying potential targets and the importance of experimental analysis to confirm specificity of these target sequences.

One of the 12 *S. enterica*-specific target sequences identified in this study, within a putative gene whose product is thought to act as energizer of the type III secretion systems in *Salmonella* (Hensel et al., 1997), was used to develop a real-time 5′-nuclease PCR assay for the detection of *S. enterica* from artificially contaminated food. The specificity of the primer/probe set was further tested against 40 *Salmonella* strains and 19 pathogenic or non-pathogenic non-*Salmonella* bacteria, most of which are known to be associated with food. An MGB-TaqMan probe was used to enhance the detection specificity, while an IAC was included to indicate false negative results.

In agreement with previous reports (Malorny et al., 2004, 2007; Moore and Feist, 2007), the detection limit using the new target was 3–8 GE per assay. Sensitivity evaluation of pure *Salmonella* cultures, i.e. sensitivity in terms of cfu, was not performed as the detection limit of live *Salmonella* cells in food matrices may vary due to the complexity of food components, background flora, and relies on the DNA extraction method that is used and the volume of DNA that is used as PCR template. Instead, a brief investigation was carried out to assess the sensitivity to detect *Salmonella* in the presence of natural background flora and food components. The detection probability for the mixture with 1.30×10^2 cfu/mL *Salmonella* was 50%, and 100% at higher concentrations of cells. The number of GE that was estimated from the standard curve was generally lower than the actual number of cfu that were added, probably because of the DNA loss during cell lysis and extraction. For the sample with 1.30×10^2 cfu/mL, the estimated amount was higher than the actual amount, implying additional sources of error.

In previous studies, the IAC DNA was typically cloned into a plasmid vector (Long et al., 2008; Malorny et al., 2003, 2004), which allows simple storage of the IAC as a recombinant DNA or a recombinant microorganism. As this strategy often requires multi-step PCR amplification and additional purification, it can be quite laborious and might bring in additional variation. An alternative strategy, employed here, is to commercially obtain synthesized single-stranded DNA and use it directly as a template for PCR. This approach has the advantage of consistent quality and availability (Hoorfar et al., 2004). Burggraf and Olgemoller (2004) successfully applied single-stranded DNA IACs in detecting multiple pathogens, but the rapidity of detection was compromised by a required melting point analysis step. In our study, synthesized single-stranded DNA was coupled with an IAC probe obtained by random shuffling of target probe, which reduced detection time by enabling the IAC signal to be generated simultaneously with the target signal. Admittedly, the cost of detection increased due to the need of an additional IAC probe. However, the random shuffling of target probe ensured the same length and GC content of the IAC product, and benefited the analysis of the duplex PCR system by enabling an equal efficiency between target and IAC amplification. It is not surprising that failure of IAC amplification was observed in the presence of high concentrations of target DNA with a competitive IAC strategy. However, IAC amplification is not necessary if the target is amplified, as the goal of an IAC is to reduce the likelihood false negative results due to the presence of PCR inhibitors (Hoorfar et al., 2004).

The successful detection of *S. enterica* in three artificially contaminated foods within 10 h was achieved using the described method. The rare failure of detection in these samples was caused by the errors in DNA extraction and PCR inhibitors that may have been derived from the food matrix. Although quantitation of genome copies in a sample is an additional benefit of the real-time PCR, at present, it can only provide an estimate of the number of GE present. Variability might be introduced due to the degradation of standard DNA, differences in PCR efficiencies between unknown samples and standards, variations in DNA extraction from a complex food matrix, etc.

In conclusion, a novel comparative genomics approach for identifying specifies-specific nucleotide sequences that are not confined to known functioning genes was used to identify *S. enterica*-specific target sequences. Several nucleotide targets were investigated and one target sequence within the *ssaN* gene was used to develop a real-time PCR for the detection of *S. enterica* in artificially contaminated foods. Additional studies are planned to include more bacterial strains for the evaluation of the specific targets, and using this strategy, it is conceivable the unique targets could be identified for detection of any bacterium for which a genome sequence is available.

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All authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijfoodmicro.2009.12.004.

References

Abubakar, I., Irvine, L., Aldus, C.F., Wyatt, G.M., Fordham, R., Schelenz, S., Shepstone, L., Howe, A., Peck, M., Hunter, P.R., 2007. A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. Health Technol. Assess. 11, 1–216.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Anonymous, 2006. Burden and causes of foodborne disease in Australia: annual report of the OzFoodNet network, 2005. Commun. Dis. Intell. 30, 278–300.

Anonymous, 2007. Multistate outbreak of *Salmonella* serotype Tennessee infections associated with peanut butter—United States, 2006–2007. Morb. Mortal. Wkly. Rep. 56, 521–524.

Anonymous, 2008. Isolation And Identification of *Salmonella* From Meat, Poultry And Egg Products (MLG 4.04). United States Department of Agriculture, Food Safety and Inspection Service.

Anonymous, 2009. Multistate outbreak of *Salmonella* infections associated with peanut butter and peanut butter-containing products—United States, 2008–2009. Morb. Mortal. Wkly. Rep. 58, 85–90.

Burggraf, S., Olgemoller, B., 2004. Simple technique for internal control of real-time amplification assays. Clin. Chem. 50, 819–825.

Chen, C.Y., Nace, G.W., Irwin, P.L., 2003. A 6×6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. J. Microbiol. Methods 55, 475–479.

Dinjus, U., Hanel, I., Muller, W., Bauerfeind, R., Helmuth, R., 1997. Detection of the induction of *Salmonella* enterotoxin gene expression by contact with epithelial cells with RT-PCR. FEMS Microbiol. Lett. 146, 175–179.

Doran, J.L., Collinson, S.K., Kay, C.M., Banser, P.A., Burian, J., Munro, C.K., Lee, S.H., Somers, J.M., Todd, E.C., Kay, W.W., 1994. *fimA* and *tctC* based DNA diagnostics for *Salmonella*. Mol. Cell. Probes 8, 291–310.

DuPont, H.L., 2007. The growing threat of foodborne bacterial enteropathogens of animal origin. Clin. Infect. Dis. 45, 1353–1361.
Ferretti, R., Mannazzu, I., Cocolin, L., Comi, G., Clementi, F., 2001. Twelve-hour PCR-based method for detection of *Salmonella* spp. in food. Appl. Environ. Microbiol. 67, 977–978.
Guo, X., Chen, J., Beuchat, L.R., Brackett, R.E., 2000. PCR detection of *Salmonella enterica* serotype Montevideo in and on raw tomatoes using primers derived from *hilA*. Appl. Environ. Microbiol. 66, 5248–5252.
Hensel, M., Shea, J.E., Raupach, B., Monack, D., Falkow, S., Gleeson, C., Kubo, T., Holden, D.W., 1997. Functional analysis of *ssaJ* and the *ssaK/U* operon, 13 genes encoding components of the type III secretion apparatus of *Salmonella* Pathogenicity Island 2. Mol. Microbiol. 24, 155–167.
Hoorfar, J., Malorny, B., Abdulmawjood, A., Cook, N., Wagner, M., Fach, P., 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. J. Clin. Microbiol. 42, 1863–1868.
Kim, H.J., Park, S.H., Lee, T.H., Nahm, B.H., Chung, Y.H., Seo, K.H., Kim, H.Y., 2006. Identification of *Salmonella enterica* serovar Typhimurium using specific PCR primers obtained by comparative genomics in *Salmonella* serovars. J. Food Prot. 69, 1653–1661.
Kim, H.J., Park, S.H., Lee, T.H., Nahm, B.H., Kim, Y.R., Kim, H.Y., 2008. Microarray detection of food-borne pathogens using specific probes prepared by comparative genomics. Biosens. Bioelectron. 24, 238–246.
Kubota, K., Iwasaki, E., Inagaki, S., Nokubo, T., Sakurai, Y., Komatsu, M., Toyofuku, H., Kasuga, F., Angulo, F.J., Morikawa, K., 2008. The human health burden of foodborne infections caused by *Campylobacter*, *Salmonella*, and *Vibrio parahaemolyticus* in Miyagi Prefecture, Japan. Foodborne Pathog. Dis. 5, 641–648.
Lampel, K.A., Keasler, S.P., Hanes, D.E., 1996. Specific detection of *Salmonella enterica* serotype Enteritidis using the polymerase chain reaction. Epidemiol. Infect. 116, 137–145.
Liu, Z.M., Shi, X.M., Pan, F., 2007. Species-specific diagnostic marker for rapid identification of *Staphylococcus aureus*. Diagn. Microbiol. Infect. Dis. 59, 379–382.
Long, F., Zhu, X.N., Zhang, Z.M., Shi, X.M., 2008. Development of a quantitative polymerase chain reaction method using a live bacterium as internal control for the detection of *Listeria monocytogenes*. Diagn. Microbiol. Infect. Dis. 62, 374–381.
Lu, G., Jiang, L., Helikar, R.M., Rowley, T.W., Zhang, L., Chen, X., Moriyama, E.N., 2006. GenomeBlast: a web tool for small genome comparison. BMC Bioinformatics 7 (Suppl 4), S18.
Lynch, M., Painter, J., Woodruff, R., Braden, C., 2006. Surveillance for foodborne-disease outbreaks—United States, 1998–2002. MMWR Surveill. Summ. 55, 1–42.
Malorny, B., Hoorfar, J., Bunge, C., Helmuth, R., 2003. Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl. Environ. Microbiol. 69, 290–296.
Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., Helmuth, R., 2004. Diagnostic real-time PCR for detection of *Salmonella* in food. Appl. Environ. Microbiol. 70, 7046–7052.
Malorny, B., Bunge, C., Helmuth, R., 2007. A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. J. Microbiol. Methods 70, 245–251.
McClelland, M., Sanderson, K.E., Spieth, J., Clifton, S.W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., Sun, H., Florea, L., Miller, W., Stoneking, T., Nhan, M., Waterston, R., Wilson, R.K., 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature 413, 852–856.
Moore, M.M., Feist, M.D., 2007. Real-time PCR method for *Salmonella* spp. targeting the stn gene. J. Appl. Microbiol. 102, 516–530.
Ou, H.Y., Ju, C.T., Thong, K.L., Ahmad, N., Deng, Z., Barer, M.R., Rajakumar, K., 2007. Translational genomics to develop a *Salmonella enterica* serovar Paratyphi A multiplex polymerase chain reaction assay. J. Mol. Diagn. 9, 624–630.
Rahn, K., De Grandis, S.A., Clarke, R.C., McEwen, S.A., Galan, J.E., Ginocchio, C., Curtiss III, R., Gyles, C.L., 1992. Amplification of an *invA* gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol. Cell. Probes 6, 271–279.
Rasmussen, R.P., 2001. Quantification on the LightCycler. In: Meuer, S., Wittwer, C., Nakagawara, K. (Eds.), Rapid Cycle Real-time PCR, Methods and Applications. Springer-Verlag, Heidelberg, pp. 21–34.
Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V., 2004. Fresh produce: a growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J. Food Prot. 67, 2342–2353.
Song, J.H., Cho, H., Park, M.Y., Kim, Y.S., Moon, H.B., Kim, Y.K., Pai, C.H., 1994. Detection of the H1-j strain of *Salmonella typhi* among Korean isolates by the polymerase chain reaction. Am. J. Trop. Med. Hyg. 50, 608–611.
Tong, Z., Cheng, S.-Y., 2003. Detection Results on *Salmonella* of 272 Food Samples in Zhejiang Province. Zhejiang J. Prev. Med. 15, 33–34.
Voetsch, A.C., Van Gilder, T.J., Angulo, F.J., Farley, M.M., Shallow, S., Marcus, R., Cieslak, P.R., Deneen, V.C., Tauxe, R.V., 2004. FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. Clin. Infect. Dis. 38 (Suppl 3), S127–S134.
Yao, J., Lin, H., Doddapaneni, H., Civerolo, E.L., 2007. nWayComp: a genome-wide sequence comparison tool for multiple strains/species of phylogenetically related microorganisms. *In Silico* Biol. 7, 195–200.
Zhang, W., Hughes, A., Wilt, G., Knabel, S.J., 2004. The BAX PCR assay for screening *Listeria monocytogenes* targets a partial putative gene *lmo2234*. J. Food Prot. 67, 1507–1511.